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Mechanisms Underlying Growth Hormone Effects in Augmenting Nitric Oxide Production and Protein Tyrosine Nitration during Endotoxin Challenge

TED H. ELSASSER, STANISLAW KAHL, CAROL MACLEOD, BENJAMIN NICHOLSON, JAMES L. SARTIN, AND CONGJUN LI

Growth Biology Laboratory, United States Department of Agriculture, Agricultural Research Service (T.H.E., S.K., C.L.), Beltsville, Maryland 20705; Cancer Center, University of California San Diego (C.M., B.N.), La Jolla, California 92093; and Department of Anatomy, Physiology & Pharmacology (J.L.S.), Auburn University, Auburn, Alabama 36849

The present study defined the effects of GH administration on components of the nitric oxide (NO)-generating cascade to account for observed increases in NO production and protein nitration after an immune challenge. Calves were assigned to groups with or without GH treatment (100 μg GH/kg body weight or placebo im, daily for 12 d) and with or without low-level endotoxin [lipopolysaccharide (LPS), 2.5 μg/kg, or placebo, iv]. Plasma was obtained for estimation of NO changes as [NO_2^- + NO_3^-] (NO_x). Transcutaneous liver biopsies were collected for measurement of protein tyrosine nitration, cationic amino acid transporter (CAT)-2 mRNA transporter, and constitutive NO synthase (cNOS), inducible NOS (iNOS), and arginase activity. Liver protein nitration increased more than 10-fold 24 h after LPS and an additional 2-fold in animals treated with GH before LPS. GH increased plasma NO_x after LPS to levels 27% greater than those measured in non-GH-treated calves. LPS increased CAT-2 mRNA after LPS; GH was associated with a 24% reduction in CAT-2 mRNA content at the peak time response. cNOS activity was 3-fold greater than iNOS after LPS. NOS activities were increased 140% (cNOS) at 3 h and 169% (iNOS) at 6 h, respectively, after LPS; GH treatment increased cNOS activity and the phosphorylation of endothelial NOS after LPS more than 2-fold over that measured in non-GH-treated calves. The data suggest that an increased production of nitrated protein develops in the liver during low-level, proinflammatory stress, and nitration is increased by GH administration through a direct effect on the competing activities of NOS and arginase, modulatable critical control points in the proinflammatory cascade. (*Endocrinology* 145: 3413–3423, 2004)

MEASURES EVOKED DURING the onset of an immune stress such as endotoxemia serve to redirect physiological priorities toward those deemed more necessary for survival (1, 2). The magnitude of redirection is largely proportional to the severity of the insult. Important to the cycle through which homeostatic balance is initiated and restored is the well-timed elaboration of proinflammatory and counterinflammatory cytokines and the compartmentalized evolution of reactive nitrogen free-radial ($NO^\bullet$) and oxygen intermediates (superoxide anion), which influence a redistribution in organ blood flow and orchestrate the flux in metabolic hormones required to facilitate or retard tissue utilization of specific nutrients (3, 4). Nitric oxide (NO) is produced through the cleavage of the guanidino-amino-N of arginine and condensation of this N with oxygen by the four nitric oxide synthase (NOS) isoforms (constitutively present, low-level output endothelial, neuronal, and mitochondrial isoforms), as well as a high-output inducible isoform, during immune challenge (5, 6). One of the critical features of NO biology, however, is not the extent to which NO is formed, but rather how it interacts within components in the cell. Important to what and how NO influences biological functions is the prevailing redox environment in the cell. Normal protein functions are impaired when the respective proteins are nitrated. Specific pathological associations have been documented (7, 8) in which conditions are such that NO reacts with the superoxide anion to form the highly reactive tyrosine-nitrating moiety peroxynitrite ($ONOO^-$; Refs. 9 and 10). Consideration of tissue pathology has been correlated to immunohistochemical detection of nitrated proteins using antibodies to nitrotyrosine (11, 12).

Some of the goals of immune response intervention strategies are to limit morbidity, break cycles of progressive tissue damage leading to multiorgan failure, and stop mortality. Pharmacological intervention strategies particularly relevant to Gram-negative sepsis rely on the use of antibiotics to kill pathogens, and many of these strategies involve the use or potential use of adjunct treatments to further stabilize metabolism. The stabilization and reversal of net protein nitrogen loss is considered important to and reflective of a positive prognosis for recovery (13, 14). Over the past few years, exogenous GH treatment has been explored as one such potential adjunct for management of catabolic processes that further challenge the host response to infection stress. Research suggested that GH might have positive effects on the host response to infection stress based on reports of improved immune function in which GH was administered to

Abbreviations: BW, Body weight; CAT, cationic amino acid transporter; cNOS, constitutive NOS; eNOS, endothelial NOS; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; iNOS, inducible NOS; LPS, lipopolysaccharide; NO, nitric oxide; NOS, nitric oxide synthase; NO_x, NO_2^- + NO_3^- (the stabile oxidation decay products of NO); $ONOO^-$, peroxynitrite; XO, xanthine oxidoreductase.

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animals. For example, some data suggested that GH treatment increased survival of experimental animals infected with *Salmonella* sp. or *Escherichia coli* (15–17), perhaps in conjunction with more efficient elimination of the pathogen. However, specific concerns about increased negative outcome associated with the administration of GH to patients during critical illness (18), as well as situationally poor outcomes in experimental models (19, 20), have prompted a reevaluation and termination of the practice in clinical scenarios. Interestingly, this view is occasionally countered with reports of an absence of adverse effect (21) or beneficial effect use in clinical and experimental venues (22–24). Whereas the literature documents the extreme situation in a relatively limited case occurrence of severe illness contemporary with GH treatment that resolved with mortality, there are few data, if any, that address the impact of GH administration on host response to the much more frequently encountered low-level proinflammatory stress.

In the present study, we specifically focused on the modulation of NO production and the development of protein tyrosine nitration as a reaction to a low-level endotoxin [lipopolysaccharide (LPS)] challenge to suggest pivotal locations in the host response pathway in which the impact of GH might have influence on biochemical components central to the generation of reactants needed to produce tyrosine nitration *in vivo*.

Materials and Methods

Animals

Animals used in this study were young (7–9 months old), sexually immature Angus x Hereford calves born and reared at the United States Department of Agriculture (USDA) beef cattle nutrition location at Beltsville, Maryland. Calves were the target species of choice because of their sensitivity to endotoxin, which more closely approaches that of the human compared with the rat or mouse (25). All protocols and procedures for this research were approved by the USDA Beltsville Animal Care and Use Committee, and the use of LPS and GH was governed by an investigational new animal drug application filed with and approved by the Center for Veterinary Medicine, U.S. Food and Drug Administration. Animals were housed in individual pens and fed and watered daily *ad libitum*. At the time of use, animals were in a fully anabolic state, gaining an average of 1.27 kg live weight per day and presenting mean plasma IGF-I levels of 186 ± 14 ng/ml. When used in study, each calf was acclimated to close human handling and gentle halter restraint in its own pen; 1 d before immune challenge and blood sampling, a sterile Teflon cannula (Abbocath-T, 14-gauge, Abbott Laboratories, North Chicago, IL) was inserted into the jugular vein and secured in place. Jugular cannula insertion was performed under local anesthesia using an ethyl chloride topical refrigerant spray (Gebauer Chemical Co., Cleveland, OH). After the conclusion of each sampling period, calves were returned to the nutrition herd.

LPS challenge, blood sampling, and liver biopsy

Immune endotoxin (LPS) challenge was imposed by the iv administration of LPS (*E. coli*, 055:B5, 2.5 μg/kg live weight; Sigma Chemical Co., St. Louis, MO). This level of LPS was previously shown in our laboratory to produce a transient rise in rectal temperature and an increase in plasma NO_2^- + NO_3^- (NO_x) (25). The duration of the hypoglycemic portion of the biphasic glucose response and the elaboration of acute phase response proteins indicated that physiological responses and clinical signs at this level of challenge were modulatable by a variety of otherwise normal physiological attributes such as feed intake level or stage of the estrous cycle and metabolic hormonal status (Elsasser, T. H., S. Kahl, and J. L. Sartin, unpublished observations). Samples were collected into EDTA and centrifuged, and the resulting plasma was stored frozen at −25 C.

Liver tissue samples were obtained under local anesthetic block (lidocaine 0.5%, descending tissue infiltration; Butler Co., Columbus, OH) by intercostal transcutaneous biopsy as previously detailed in Ref. 25. Three 25- to 30-mg biopsy cores were obtained using the Tru-cut biopsy needle (14-gauge, Allegiance Healthcare Corp., McGraw Park, IL) for each animal at specified time points. Samples were either frozen in liquid nitrogen and stored at −85 C or fixed overnight in 4% paraformaldehyde in PBS, transferred to 70% ethanol, and processed by paraffin embedding for sectioning at 6 μm for immunohistochemical evaluation.

Two experimental protocols were followed for the LPS challenge studies. In the first protocol, 20 calves were assigned to treatment groups (n = 5 per group) consisting of control (saline; no LPS, no GH), GH treated [recombinant bovine GH, Monsanto Inc., St. Louis, MO; 0.1 mg/kg body weight (BW), im, daily for 12 d; no LPS], LPS challenged (no GH), and GH treated plus LPS challenged. LPS was administered as two separate challenges separated by 5 d (26). GH treatments continued for the 5-d period between LPS challenges. All solutions instilled into test animals were prepared from sterile pyrogen-free solutions commercially procured (Abbott Laboratories). Single time point biopsy cores were obtained 24 h after the second LPS challenge. Data for Fig. 1 were derived exclusively from samples obtained with this dual challenge protocol.

To better evaluate potential mechanisms underlying the GH effect on NO and protein nitration and the temporal relationships between the biomarkers studied, a second investigation collected both blood and liver tissue samples as a function of time after a single LPS challenge, again administered at the 2.5 μg/kg dose. Animals (n = 24) were divided into GH and non-GH treatment groups (n = 12 per group) in a factorial arrangement of GH treatment (+/−) and biopsy sampling time; blood and liver biopsy sampling relative to the administration of the LPS challenge occurred at 0, 3, 6, and 24 h (n = 6 per time point). GH treatment (0.1 mg/kg BW, im) was administered for 12 d before sampling and LPS challenging. Three GH-treated and three non-GH-treated calves were used at each time point. Blood or tissue biopsy samples were prepared for storage or analysis as described above.

Quantitative nitrated (nitrotyrosine) protein immunohistochemistry

A quantitative measure of hepatic protein nitration (as a protein-associated nitrotyrosine epitope) resulting from the nitrooxidative stresses of LPS challenge was established using immunohistochemical staining procedures with rabbit antinitrotyrosine as the primary antibody (kindly provided by Dr. José Rodrigo, Institudo de Ramon ý Cajal, Madrid, Spain) (12). The specificity of antigen recognition was validated through a series of quality control procedures as suggested by Gow *et al.* (27), in which the primary antibody reactivity was shown to be blocked by preincubation with 10 mM 3-nitrotyrosine or $ONOO^-$-nitrated BSA (kindly donated by Dr. J. Hinson, University of Arkansas, Fayetteville, AR) as applied to tissue sections from animals previously identified as positive for nitrotyrosine staining. All relevant tissue samples were batch-processed in a single run to eliminate interprocedural daily variation in results. Three biopsy cores were collected from each animal, fixed, embedded in paraffin, and analyzed for immunoreactivity. Two section slices were mounted per slide, and the continuity of staining was compared for quality control purposes. Tissue sections were deparaffinized in xylene, treated with methanolic peroxide to eliminate endogenous peroxidase activity, rehydrated to water through progressively decreasing ethanol concentrations, and transferred to 0.05 M Tris, 0.135 M saline (pH 7.5). Sections were treated for 10 min with 0.05% Triton X-100, washed in Tris, and treated for 1 h with 3% normal goat serum in 1% casein-Tris-saline (Bio-Rad Laboratories, Hercules, CA) to block nonspecific binding. Antinitrotyrosine serum was applied at optimized (1:2500) and supraoptimal (1:5000) dilutions to sequential serial sections of tissue specimens from individual animals mounted on a single slide to extend the capacity to differentiate signal strength as a function of treatment in which the antigen was present but in low yield (27). Signal extinction occurred at 1:10,000 dilution (data not shown). Visualization of immunostaining was accomplished using the avidin-biotin-horseradish peroxidase (ABC method) complex method (Vector Laboratories, Burlingame, CA). Nuclei were counterstained for 2 min using Curazzi's hematoxylin. After dehydration of sections through

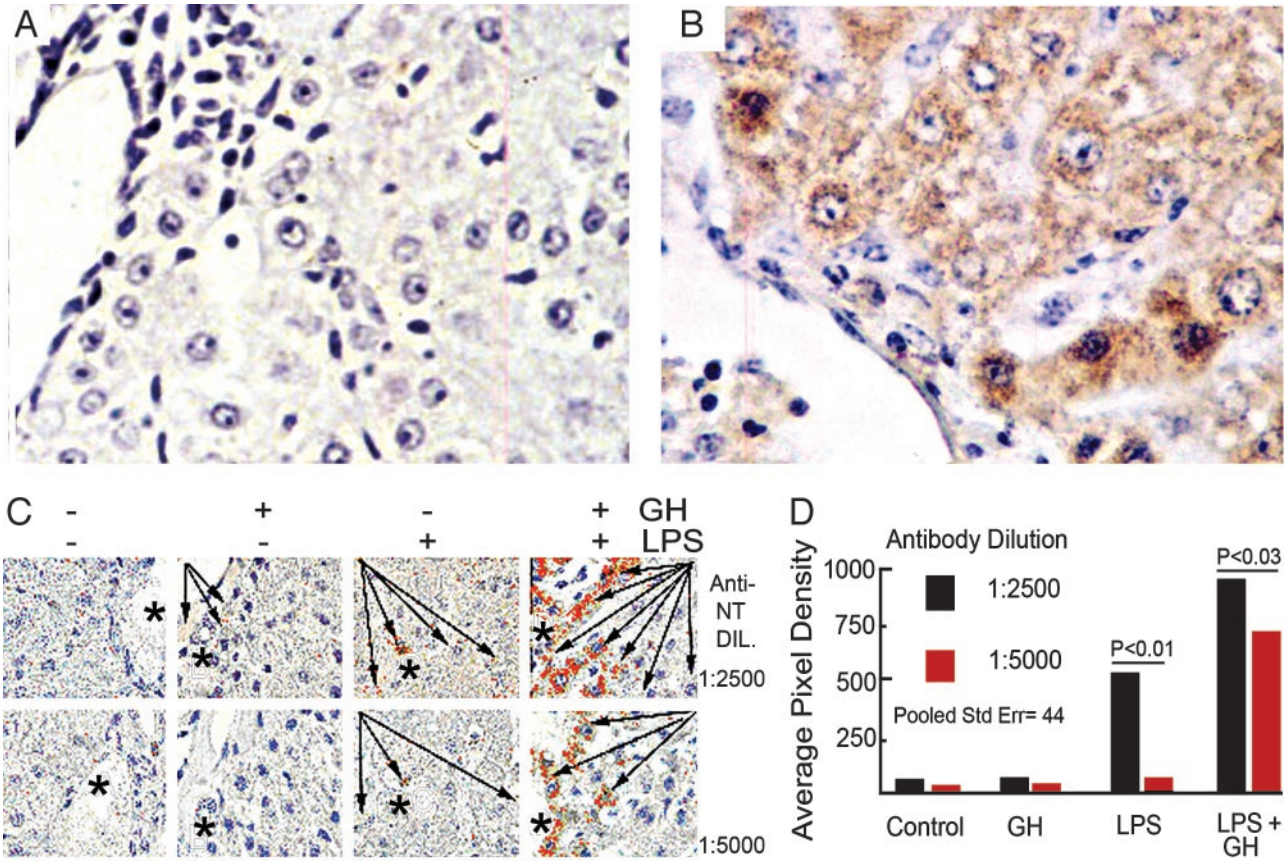

FIG. 1. Immunohistochemical localization of protein nitration in liver tissue collected from calves 24 h after a repeated saline (control) or endotoxin challenge (*E. coli*, 055:B5, 2.5 μg/kg, iv). A, Tissue from control animals displayed little or no evidence of protein nitration (100× objective). B, After LPS challenge, perivenous regions displayed highest levels of protein nitration (24 h after second challenge; 100×). C, Consecutive serial sections of tissue specimens were immunostained using two different dilutions of rabbit antinitrotyrosine (40×). *Arrows* indicate typical sites of nitration. *Asterisks within panels* indicate landmark reference points for relevance of staining characteristics in the serial sections for the indicated experimental treatment, − or +. The *bright red* signal corresponds to image analysis software-identified NT-positive pixels. D, Average color spectrum-specific pixel densities at two antibody dilutions for nitrotyrosine localization quantification as affected by LPS and GH treatments (n = 5 animals per treatment).

100% ethanol into xylene, coverslips were applied using optically clear Permaslip liquid mounting media (Alban Scientific, Inc., St. Louis, MO). Each section was photographed at full illumination power using the 40× objective of an Olympus BX-40 microscope (Olympus, Melville, NY) in two separate locations in each of the three biopsy cores using an Olympus DP-70 digital camera. Each 12.8-megapixel file was analyzed using the Image-Pro Plus Image Analysis Software (version 4.5.1, Media Cybernetics Inc., Silver Spring, MD) through a standardized protocol. Captured images were equalized in terms of contrast, brightness, and gamma using the software-driven internal best-fit equalization algorithm. The intensity of diaminobenzidine color-specific staining was obtained by defining through color cube-based segmentation a spectrum-specific range of wavelengths, hues, and intensities that corresponded to those color attributes detectable by the same staining procedure applied to an internal control liver specimen known to contain positive nitrotyrosine staining. By using serial sections of this internal standardized tissue and effecting a decreasing signal intensity to extinction with the use of progressively increasing dilutions of the primary antibody (supraoptimal dilution) (28, 29), we could accurately define and quantify signal intensity and differentiate true signal from background on the immunostained slides. Specificity of staining was further defined in the software application cutoff criteria in which object counts lower than a 3-by-3 pixel unit were eliminated. As defined in this manner, this analytical solution was found to be most commonly associated with false-negative staining, thus minimizing errors of false-positive inclusion through this conservative rubric. This spectral information was filed into a retrievable *.rge format that was subsequently used for analyzing all images. In this fashion, unbiased continuity of analysis across tissue sections was achieved; each estimate of tissue nitrotyrosine staining intensity for an individual animal or time point was obtained as the average of the summated pixels of the six pictures captured for each slide section stained at a given antibody dilution. Thus, for the two applicable antinitrotyrosine dilutions, the sum total of captured images subjected to digital processing was 12.

Plasma NO_x determination

Plasma concentrations of nitrate and nitrite (NO_x, the stabile oxidation decay products of NO) were measured as an estimate of overall NO production subsequent to the applied LPS challenges (30). Plasma NO_2^- was measured by the Griess reaction after conversion of nitrate to nitrite by *Aspergillus* nitrate reductase, as previously described (25).

Cationic amino acid transporter (CAT)-2 mRNA determination

The effect of LPS and GH treatments on the expression of CAT2/CAT2a was assessed by Northern blotting using the mouse sequences for the CAT-2 class transporters and protocols, as described previously by Nicholson *et al.* (31). In brief, total RNA from bovine liver tissue was prepared and extracted with TRIzol (Gibco BRL, Life Technologies, Grand Island, NY). RNA (10 μg total per lane) species were separated on denaturing formaldehyde gels. Nucleotide size markers (Gibco BRL, Life Technologies) were used to determine the relative size of each

message. The RNA was transferred overnight onto a Magnacharge filter (Micron Separations Inc., Westborough, MA). Cross-linking was accomplished with UV light and by baking the blot in a vacuum oven for 2 h. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and CAT-2 cDNA fragments were random-primed labeled using 10^6 cpm/ml [α-^{32}P]dCTP. Filters were treated, prehybridized, and hybridized using standard protocols. After high stringency washing, filters were exposed, and the films were processed for densitometric analysis. Gel loading was assessed using GAPDH hybridization. Densitometric assessment of specific CAT-2 and GAPDH bands was performed using a Chem-Imager (Alpha Innotech Corp., San Leandro, CA) in scanning 1-D multiline mode. CAT-2 values were corrected for loading variation by normalizing to the corresponding GAPDH density value.

Western blot protein analysis

Tissue homogenates of liver biopsy samples collected at 0, 3, 6, and 24 h after a single LPS challenge at 2.5 μg/kg BW were used to assess by Western blot the temporal relationship between changes in NOS isoforms and the generation of nitrated proteins. Constitutive NOS (cNOS) and inducible NOS (iNOS) isoforms, nitrated proteins, and the phosphorylation-activated ($_{1177}$phosphoserine)-endothelial NOS (eNOS) were measured using antibodies against eNOS (rabbit antibovine type 3 NOS, 0.5 μg/ml; Upstate Biotech Inc., Lake Placid, NY), rabbit antimouse type 2 macrophage iNOS (1:1000 dilution, Upstate Biotech, Inc.), rabbit antinitrotyrosine (1:1000; Dr. José Rodrigo, Madrid Spain), and mouse monoclonal antiphospho-eNOS (1:2000; Biomol International, Plymouth Meeting, PA), respectively. For the purpose of the present study, the terms eNOS and cNOS are used interchangeably because eNOS in the major liver isoform of the cNOS class of enzymes and the available commercial antibody against eNOS was highly cross-reactive in the bovine species. Liver homogenates were prepared by dispersing biopsy cores in protease inhibitor-stabilized HEPES buffer, as will be further described in the differential NOS enzyme assay section in this paper. The supernatant of a 14,000 $\times$ *g* centrifugation preparation was obtained and diluted according to protein content to achieve a concentration of 2 μg protein/μl buffer. Homogenates were mixed 1:2 with sodium dodecyl sulfate-Laemmli buffer, boiled 5 min, and centrifuged at 10,000 $\times$ *g* for 2 min; and 20 μg protein was loaded onto 10% acrylamide gels (Bio-Rad Laboratories). Proteins were separated under nonreducing conditions in a 120-V field for 75 min, transferred to nitrocellulose overnight at 20 V, and then probed with the respective concentrations of antibodies after blocking of nonspecific binding with 3% BSA (Sigma Chemical Co.) in Tris-saline containing 2% filtered normal goat serum. Proteins were resolved and quantified by autoradiographic densitometry of bands produced on XOMAT-AR film (Kodak Inc., Rochester, NY) by standard chemiluminescent reporter technology. For Western blots probed with anti-iNOS and anti-eNOS, band intensities were estimated as a pixel volume density using image analysis values obtained with a Chem-Imager (Alpha Innotech Corp.). For the nitrotyrosine and phosphorylated-eNOS Western blot analysis, the mean OD of a gel lane was obtained by scanning the radiographic film into a tagged image file (*.tif) format and analyzing the image using the line profile OD operations with background correction as per the Image-Pro Plus Image Analysis Software (version 4.5.1, Media Cybernetics Inc.). An abbreviated immunodot-blot protocol was performed (data not presented) to confirm results of the effects of LPS and GH treatment on protein tyrosine nitration obtained with the standard Western blot protocol described. For the dot-blot, 2-μg protein homogenate samples were applied in triplicate, dried onto nitrocellulose membranes, and probed with the antinitrotyrosine antibody at 1:1000; for this application, a fluorescent reporter (donkey-antirabbit IgG-Alexa 488, Molecular Probes, Eugene, OR) was used, and the signal was read using a scanning laser fluorimager (Typhoon 9400, Amersham Biosciences, Piscataway, NJ). For the dot-blot, preincubation of antinitrotyrosine serum with 10 mM 3-nitrotyrosine eliminated all but the strongest signal dots.

Differential NOS enzyme activity determination

Activity of different isoforms of NOS was differentiated and determined by analyzing the conversion of [^{3}H]arginine to [^{3}H]citrulline in the presence and absence of isoform-specific NOS inhibitors according to a modified method of Salter *et al.* (32) adapted for bovine liver samples as previously described (25). In brief, liver biopsy samples were homogenized (1:5, wt/vol) using a microcentrifuge pestle (Kontes, Vineland, NJ) in 50 mM HEPES (pH 7.4) containing 320 mM sucrose, 1 mM dithiothreitol, 1 mM EDTA, and protease inhibitor cocktail for mammalian cell extracts (Sigma Chemical Co.; 0.01 ml/ml buffer). After centrifugation (30 min at 100,000 $\times$ *g*), the supernatant was incubated for 30 min at 37 C with 18 nM L-[2,3-^{3}H]arginine (36.8 Ci/mmol; DuPont-NEN Life Science Products, Boston, MA) in 50 mM HEPES (pH 7.4) containing 1 mM dithiothreitol, 4 μM flavin adenine dinucleotide, 4 μM flavin mononucleotide, 0.2 mM reduced nicotinamide adenine dinucleotide phosphate, 50 μM tetrahydrobiopterine (ICN Biomedicals, Inc., Aurora, OH), and 25 μM L-arginine. Valine (50 mM), citrulline (1 mM), and ornithine (1 mM) were included in the reaction mixture to inhibit activity of arginase and other enzymes of the urea cycle that might compete for the substrate arginine. The reaction was terminated by the addition of Dowex AG50WX-8, 200–400 Mesh Na^+ resin (Bio-Rad Laboratories), and L-[2,3-^{3}H]citrulline was quantified in the supernatant by liquid-scintillation counting. Activity of cNOS was expressed as citrulline formation (picomoles per minute per milligram) in the presence of calcium-chelating EGTA or the calmodulin blocker trifluoperazine (Sigma Chemical Co.). Activity of iNOS was expressed as citrulline formation (picomoles per minute per milligram protein) that could be inhibited by S-methylisothiourea sulfate (1 mM), the selective inhibitor of iNOS (33), in the presence of EGTA (1 mM). Protein concentration was determined with bicinchoninic acid reagent and BSA as a standard (Pierce Chemical Co., Rockford, IL).

Tissue arginase assay

Liver tissue content of arginase activity was measured by the rate of conversion of arginine to urea by protein content-normalized, HEPES-buffered tissue homogenates under basal and Mn^{2+}-activated conditions, as previously described (34).

Statistical analysis

Data were statistically analyzed by ANOVA with the general linear models procedure of the Statistical Analysis System (version 6.1; SAS Institute, Cary, NC) as previously used (26). Data are presented as either means $\pm$ SEM or as means with the estimate of variance stated as the pooled SE of the linear model (used graphically for clearer presentation). Where appropriate, responses collated over time were analyzed in regard to a response variable calculated as an area under the curve generated from concentration $\times$ time integration by simple trapezoidal summation. Individual group or time point differences were assessed using specific contrast statements in the statistical model; differences were significant when $P < 0.05$.

Results

Tissue levels of protein nitration, as detected immunohistochemically by staining for the presence of protein-bound nitrotyrosine, were increased significantly in tissues obtained from calves 24 h after their second exposure to LPS in the 5-d rechallenge protocol (Fig. 1). The pattern of staining was remarkably different after LPS challenge according to whether animals had been treated with GH or not. The generalized diffuse pattern of staining noted in animals challenged only with LPS (no GH) was intensified in animals treated with GH before LPS, with very discrete cell-to-cell changes in staining that followed a decreasing gradient pattern in pericentral veinous areas in which the most intense staining in cells was under the 100$\times$ oil objective in those cells immediately adjacent to the central vein. Liver cells of GH-treated calves challenged with LPS displayed increased nitrotyrosine staining compared with non-GH-treated calves when the primary antibody was used at 1:2500. This staining was proportionately decreased in sections from LPS-treated calves at this supraoptimal dilution, further demarcating

differences in tyrosine nitration after LPS in GH-treated and -nontreated calves. Relative mean summated staining intensities in pixel density units were 46, 52, 556, and 969 at 1:2500 and 19, 20, 47, and 759 (pooled SE = 44) at 1:5000 for control, GH-treated, LPS-challenged, and LPS-challenged/GH-treated calves, respectively. The effect of GH to increase liver nitrotyrosine staining after LPS challenge was significant ($P < 0.05$ at 1:2500, $P < 0.02$ at 1:5000).

To better evaluate the nitration response as a function of time after a single LPS challenge and GH treatment, a Western blot analysis of protein tyrosine nitration of liver homogenate proteins was performed, and the results were presented in Fig. 2. As shown in Fig. 2A, particularly intense immunostaining for nitrotyrosine, the indicator of protein nitration, was evident in a variety of different proteins of varying molecular weight. A low-level signal was present in samples obtained from both control and GH-treated calves. The intensity of the signal increased after the administration of LPS to calves and peaked at the 24-h point in sampling. Mean OD of lanes associated with the various treatments and times (Fig. 2B) indicated that the generation of the nitrotyrosine signal occurred by 3 h after LPS challenge. Protein tyrosine nitration was increased 3 h after LPS administration in homogenate preparations from GH-treated calves, whereas a significant increase in tyrosine nitration in non-GH-treated calves was first observed at the 6-h sampling point ($P < 0.03$, *vs.* time zero values in both GH-treated and non-GH-treated calves; $P < 0.02$, significantly earlier in GH-treated calves). Overall, LPS administration increased liver protein tyrosine nitration through the 24-h sampling point ($P < 0.0001$), and this effect was further amplified by GH treatment ($P < 0.002$).

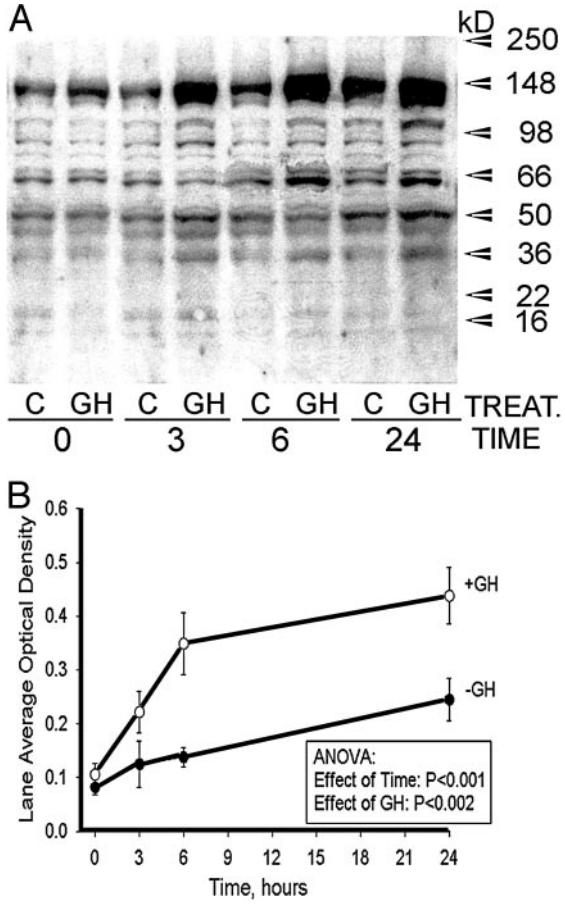

FIG. 2. Western blot analysis of liver homogenate proteins (20 μg protein/lane) separated by PAGE and probed on nitrocellulose using 1:1000 rabbit antinitrotyrosine. The profile of electrophoretically separated proteins immunostained for nitration is presented in panel A. Data in panel B represent OD means for 24 animals divided equally (n = 3 per time point) among control and GH treatments with biopsy specimens obtained from individual animals at 0, 3, 6, and 24 h relative to the administration of the LPS challenge (2.5 μg/kg, iv). C, Control.

Plasma concentrations of NO_x were increased ($P < 0.02$) after LPS challenge, peaking at 6–8 h after LPS administration (Fig. 3). Basal plasma NO_x levels were higher at time zero in GH-treated calves compared with non-GH-treated calves, and the area under the concentration × time response curve was similarly greater in GH-treated calves ($P < 0.02$).

An essential step in the production of NO by NOS isoforms is the transmembrane uptake of the NOS substrate arginine. The effects of LPS challenge and GH treatment on the induction of mRNA for CAT-2 transporter are presented in Fig. 4. Fig. 4A is representative of the measured mRNA levels in individual animals from 0–6 h after LPS. Fig. 4B represents mean densitometry levels by group and time through the 24-h biopsy point. LPS challenge resulted in a progressive increase in tissue CAT-2 mRNA that peaked 6 h after LPS and returned to basal levels by 24 h post LPS. GH treatment had no effect on basal levels of CAT-2 mRNA; CAT-2 mRNA levels were lower ($P < 0.05$) at 6 (24%) and 24 h (78%) post LPS in GH-treated compared with non-GH-treated calves.

The effects of GH treatment on liver arginase, the major enzyme competing with NOS for substrate arginine availability and utilization, are presented in Fig. 5. Manganese activation resulted in a 4-fold increase in arginase-based production of urea from arginine. Under either state of activation, GH significantly decreased arginase conversion of

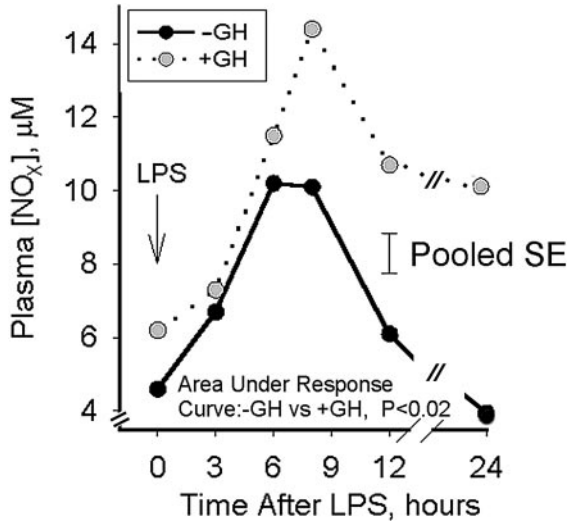

FIG. 3. Plasma concentrations of NO_x (nitrate + nitrite) as a measure of NO production in control (−GH) calves and calves treated with recombinant bovine GH (0.1 mg/kg, im, × 12 d) before challenge with LPS (*E. coli*, 055:B5, 2.5 μg/kg, iv). Values represent mean determinations for n = 3 per treatment and time.

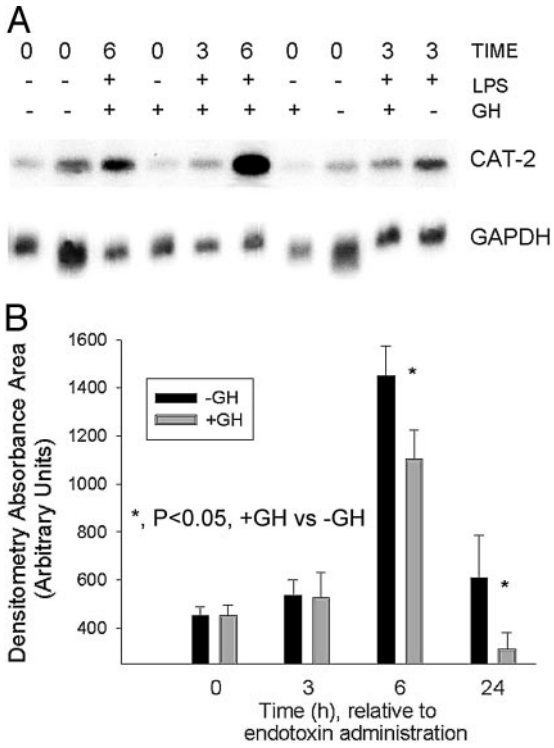

FIG. 4. Measurement of mRNA expression over time after LPS challenge (*E. coli*, 055:B5, 2.5 μg/kg, iv) in calves. A, CAT-2 Northern blot images associated with the GAPDH normalizing mRNA. B, Mean densitometry values (arbitrary density absorption units) for GH and control calves challenged with LPS and individually sampled via transcutaneous liver biopsy (n = 3 per treatment and time).

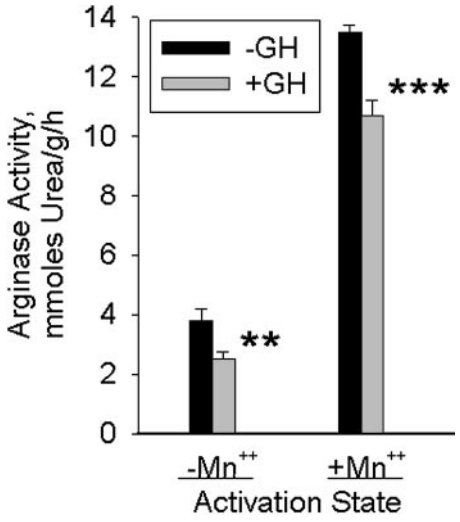

FIG. 5. Liver tissue activity of the urea cycle enzyme arginase as affected by GH treatment or saline injection. Data were collected from calves used in LPS challenge protocol 2 in which samples were collected over time after the administration of the LPS. Because there was no difference in arginase activity levels, basal or Mn^{2+}-activated, as a function of LPS challenge *per se*, the data represent the pooled means of the 12 animals at all time points as grouped by +GH or −GH treatment.**, $P < 0.02$; ***, $P < 0.01$.

arginine to urea by an average of 22% ($P < 0.05$). LPS challenge had no measurable effect on arginase activity (data not shown).

The effects of LPS and GH treatments on iNOS and cNOS isoforms as determined by Western blot are presented in Fig. 6. LPS challenge resulted in a significant increase in iNOS protein content in liver homogenates from both GH-treated and non-GH-treated calves (Fig. 6A). The overall effect of GH was a trend to maintain an elevated iNOS protein level after LPS at 6 and 24 h ($P < 0.08$) in contrast to non-GH-treated animals, in which the iNOS response clearly decreased after the peak at 3 h. cNOS protein levels, estimated as the endothelial type 3 isoform (Fig. 6B) were somewhat variable over time but were largely not affected by LPS challenge or GH treatment. Again, there was a trend for increased eNOS levels at 6 and 24 h after LPS compared with time zero, but the magnitude of the animal-to-animal variability prevented significant differences from being detectable.

When the temporal profile of cNOS and iNOS responses to LPS and GH treatment was evaluated as enzyme activity, the pattern of measured responses varied from that characterized with the Western blot technique. In Fig. 7A, iNOS activity levels increased numerically over those characterized in samples collected at time zero, but at the respective time points no values were significantly greater than those at time zero. The overall effect of LPS to increase iNOS activity as suggested by the statistical ANOVA analysis was significant when the responses at 3 and 6 h were addressed collectively. Activity levels decreased at 24 h to levels comparable with those measured at time zero; the overall effect of GH in the increase in iNOS activity was not significant. cNOS levels, measurable at time zero in all calves, were 6- to 8-fold higher than iNOS activity levels ($P < 0.005$). Statistical inferences are presented on the graph for only the relationships present at times 0 and 3 h to simplify the visual presentation. After LPS, cNOS activity was significantly increased over basal levels only in GH-treated animals ($P < 0.003$), although numerically small increases in cNOS activity could be de-

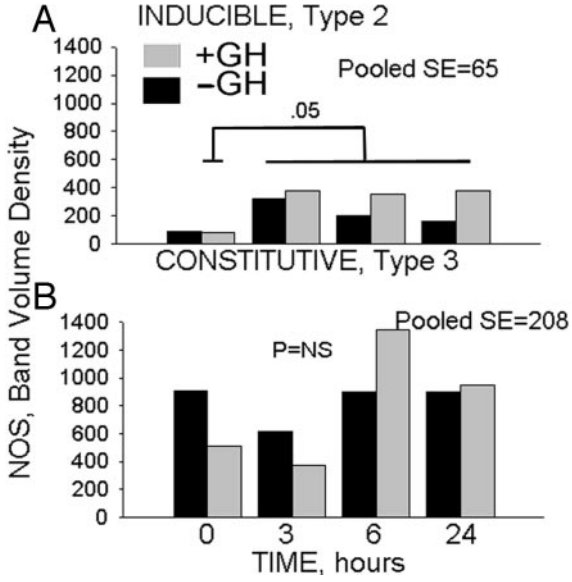

FIG. 6. Western blot analysis of changes in iNOS protein levels (A) and cNOS protein levels (B) as a function of time after LPS challenge (*E. coli*, 055:B5, 2.5 μg/kg, iv) administration. Values represent means of three calves from each treatment period for both GH-treated (+GH) and non-GH-treated (−GH) calves. NS, Nonsignificant.

tected in non-GH-treated calves ($P = 0.078$) at 3 h. At this 3-h point in response, cNOS levels of non-GH-treated calves were 54.5% of the mean activity levels measured in homogenates from GH-treated calves ($P < 0.02$). The relative iNOS and cNOS activity increases associated with LPS were variable over time; approximately 25.4% of the total substrate (arginine) conversion activity was associated with iNOS in GH-treated calves at 3 h, whereas a similar percentage of total activity was apparent in homogenate preparations from non-GH-treated calves at 6 h after LPS ($P < 0.01$; time effect of GH). In contrast to the effects of LPS on iNOS activity, cNOS activity increases were maintained at the 24-h point in both treatment groups.

The $_{1177}$serine phosphorylation profile for eNOS, as determined by Western blot, is shown in Fig. 8. The ANOVA indicated that the effect on LPS to increase the phosphorylation of eNOS was significant over time ($P < 0.0001$). The response appeared over time to have a biphasic component in which the initial effect plateaued between 3 and 6 h, but further increased at 24 h post LPS. Levels of phosphorylation were increased by GH at each individual time point including time zero (Fig. 8), resulting in an overall significant main effect of GH on the eNOS phosphorylation ($P < 0.001$).

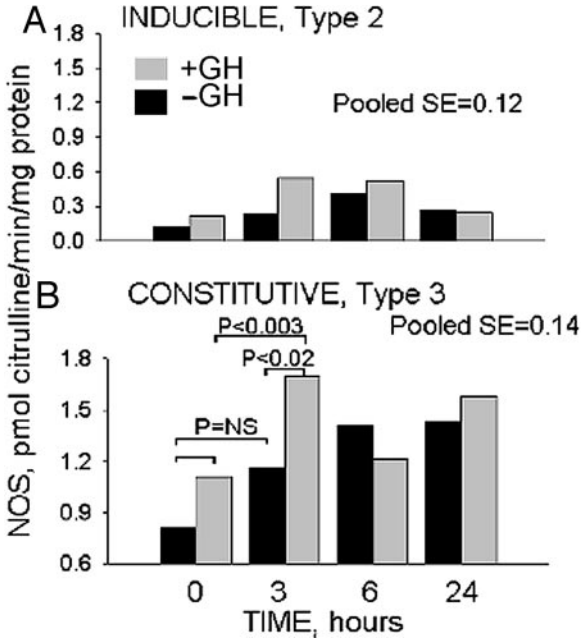

FIG. 7. iNOS (A) and cNOS (B) enzyme activities of liver homogenate biopsy samples as a function of time after LPS challenge and GH treatment status. Values represent means of three separate determinations per time period for both GH-treated (+GH) and non-GH-treated (−GH) calves.

Discussion

The presence of nitrotyrosine in tissues is considered the telltale sign that biochemical events have transpired to facilitate interactions between NO and superoxide anion that result in the production of $ONOO^-$ and accompanying post-translational chemical modification of proteins called nitration (7, 8). Low-level protein nitration has been detected under healthy basal conditions, but nitration at high levels was found more frequently in association with frank pathology (8). The breakpoint between function and disease as regards protein nitration level in tissues has yet to be established.

This study constitutes the first report of the temporal elaboration of protein nitration as affected by GH treatment after a single LPS challenge. Similarly, we have reconfirmed the prior observation that GH treatment is associated with increased NO production after the administration of a relatively low and nonlethal dose of LPS (35). The data suggest that the nitration of tyrosine residues in a variety of liver proteins occurs in a time course compatible with the increase in plasma NO_x concentrations and the increase in NOS isoform activity (in contrast to NOS tissue content as assessed by Western blot). The temporal development of protein nitration more closely fit the pattern of NOS activity changes rather than the Western blot protein data. The data further suggest that the relative activity contributions of iNOS and cNOS toward NO production appear to vary over time where the early response (*i.e.* 3 h) can be characterized as a combined effect of cNOS and iNOS (predominantly cNOS with a smaller iNOS contribution), whereas the continued response after 3 h can be accounted for by cNOS activity alone. A critical point appears to be what activity state each isoform might have been in, or have been changed to, as a function of the LPS and GH treatments. In this fashion, it was very revealing that at the level of LPS challenge imposed on these animals, the NO-generating capacity of the constitutive isoform outweighed the generating capacity of iNOS by a factor of almost three times, data not reflected in the analysis attained by NOS Western blot.

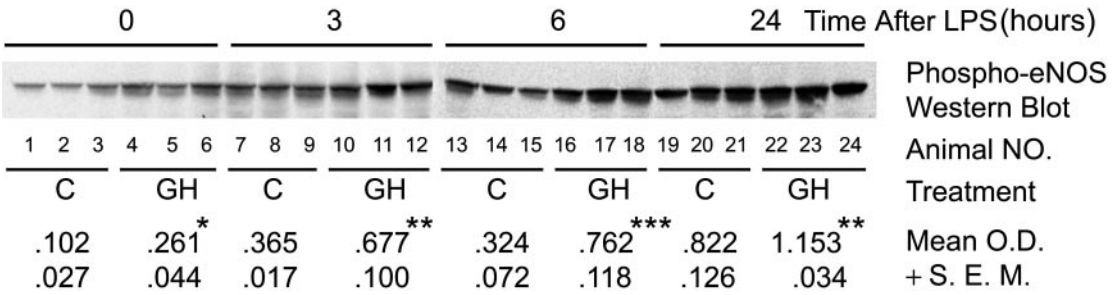

FIG. 8. Serine-specific phosphorylation patterns of liver eNOS as affected by LPS challenge (*E. coli*, 055:B5, 2.5 μg/kg, iv) in control (C) and GH-treated calves. Liver homogenates of the 24 individual calves are presented with the respective times and treatment groupings indicated by the *horizontal lines*. Values represent the mean ± SEM scanned OD for each group of three calves sampled. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

Our present data indicate that GH treatment affects both basal and LPS-induced increases in nitrate concentrations in plasma, suggestive of an effect to modulate the generation of NO at the enzymatic source. In particular regard to the higher nitrate levels in GH-treated animals before LPS challenge, our data reflect a continuity between the observed nitrate concentrations and the higher basal levels of cNOS activity. Biochemically relevant events modulating this activity can include the flux patterns of calcium, calmodulin, nicotinamide adenine dinucleotide, and, most recently described, epitope-specific directed phosphorylation and dephosphorylation of serine residues such as $_{1179\text{-bovine}/1177\text{-human}}$serine, $_{116}$serine, and $_{497}$threonine (36). Reports in the literature suggest that the phosphorylation activation of eNOS is regulated through a protein kinase B/AKT-driven kinase reaction and proinflammatory cytokines such as TNF-α, in fact, up-regulate a type-2 (inducible) NOS-independent production of NO via this AKT phosphorylation of eNOS (37) and by the fact that GH has activity to up-regulate AKT (38). GH may also increase local NO production via these eNOS routes. This modulation of eNOS activity, and therefore NO production, is further refined by intricate interactions between peroxisome proliferator-activated receptor-γ (39) and calcium conditions (40). Knowing that post-translational modification of these NOS proteins affects their function, our data are consistent with the concept that GH treatment was affecting the state of NOS activity more than the changes in actual protein levels that might have been effected by treatment(s). The data in Fig. 8 are unique in that they constitute the first observation on an effect of GH to modulate eNOS activity by alterations in this specific post-translational phosphorylation. The persistence of serine-specific phosphorylation is consistent with the measured increased levels of enzyme activity as presented in Fig. 7. Particularly relevant is the presence of the 2.5-fold increase in phosphorylation status of eNOS, 2-fold greater cNOS enzyme activity, and 50% greater plasma concentrations of NO_x at time zero relative to LPS.

The plasma levels of NO_x measured in our study contrast sharply with levels of NO_x measured in other LPS-challenge studies in which iNOS was tremendously up-regulated, NO_x reached levels approximately 200 μM, and mortality was common to the outcome (for example, see Ref. 41). Our data indicate that protein nitration can occur during relatively mild proinflammatory challenge and need not be restricted only to severe challenge or response. Our data are supported by experiments of Javeshghani and Magder (42) in which pigs that received a 20 μg/kg LPS challenge displayed protein tyrosine nitration in various tissues but had minimal, if any, change in iNOS character (mRNA, Western blot, *etc.*). Our data, perhaps, help to explain their findings in that the up-regulation of cNOS/eNOS appears to be sufficient to generate the needed nitrating species ($ONOO^-$) in a situation in which the impact of iNOS was negligible.

The facilitated transport of arginine into cells is a component necessary for intracellular NO production (31). CAT-2 mRNA levels increase after LPS challenge, but apparently only significantly so at sampling points more than 3 h after the application of the LPS. Interestingly, animals treated with GH displayed CAT-2 mRNA levels significantly lower at the peak post-LPS response point, and this may indicate that the initial increased production and flux of NO/NO_x was sufficiently accounted for by available levels of intracellular arginine and arginine transporter present at the time of the initiation of the LPS challenge. The literature suggests that the ability for GH to moderate transport of arginine into cells is highly tissue-specific, for example, varying in different parts of the gut (43). No data other than that presented here address the influence of the GH specifically on CAT-2 induction, particularly as regards changes in mRNA content shortly after LPS challenge. However, the nature of the decrease in CAT-2 mRNA 6 and 24 h after LPS challenge may represent a form of GH-mediated compensation on the part of the cell to limit overproduction of NO and nitrating agents at a time in the host response at which homeostatic equilibrations are taking place.

With particular regard to the liver, it is apparent that the enzyme complex xanthine oxidoreductase (XO) is a major source of superoxide anion, a reaction product shown to condense with NO to from $ONOO^-$ *in vivo* (44) and nitrate proteins. Recently, we demonstrated that LPS caused a significant increase in tissue and plasma XO activity levels and that concurrent GH treatment exacerbated this response (45). Concomitantly, therefore, GH administration can be associated with increased activity of the two major classes of enzymes that form the reactants leading to $ONOO^-$, superoxide anion via XO, and NO via NOS.

An important point relevant to the competitive pull on arginine for use in the urea cycle *vs.* NO production is the magnitude of substrate turnover affected by arginase in comparison to NOS isoforms. Although the effect of GH was significant, albeit small, the level of arginase activity reduction caused by GH treatment has a potentially huge impact on the relative compartmental availability of arginine to access NOS. The modulation of NO production achieved through a competition for substrate arginine between NOS isoforms and arginase has been reported previously (46), but never before addressed in regard to the potential impact of GH as presented here. Comparing arginine conversion data in Fig. 5 with that in Fig. 7, it is readily apparent that the arginase activity per unit tissue is upwards of 1000 times more active than the NOS activities. Interestingly, we have previously demonstrated that the duration of GH treatment had significant impact on the level of arginase activity and accompanying urea production from arginine (34). In that paper, we demonstrated that a short-term treatment of calves with GH for 3–5 d was without the significant effect on arginase activity levels, whereas a decrease in arginase activity was apparent with 10–12 d of GH treatment. The reason for this is not entirely clear. Thus, the relative effect of GH on urea cycle-directed consumption of arginine must be factored into the level of response through which tissue nitration might occur in endotoxemia.

This concept of differential effects and outcomes being tied to the duration of an adjunct treatment such as the use of GH in clinical cases may have significant implications on how data are interpreted with regard to a benefit or risk qualifier being assigned to GH therapy/administration in mildly ill or severely ill subjects. A launch point for concern about the use of GH in clinical sepsis was the report of increased mortality

in adults that was associated with the use of GH in critically ill patients (18). Much care should be taken in interpreting the literature-based implications of GH use during illness and the final regard for risk assessed in terms of demonstrated increased pathology in the particular application. For example, if in our future research we can ascribe a negative outcome to the increased presentation of liver nitration of isoform-specific NO generation commensurate with interactions between GH and immune stress, then for that situation GH administration would be inadvisable. Such was the case when we explored the potential use of GH to offset tissue losses in calves that were experimentally infected with parasitic pathogens known to cause cachexia and wasting (47, 48). However, our observations of increased morbidity or mortality in parasitized calves that had been treated with GH cannot be readily equated with the increased mortality rate observed by Liao *et al.* (19, 49) when rats were preconditioned with GH before a high-level LPS challenge. Many of the detrimental effects of GH in our studies were partially related to a compromised metabolic state wherein the relative lack of fat reserves in GH-treated calves constituted a condition in which animals could not mobilize fat for energy purposes during the anorexic stage of the onset of response to these infecting agents.

Results from Koea *et al.* (21) and Roelfsema *et al.* (50) indicate how GH could differentially affect physiological systems (cardiovascular function may be affected more than metabolism with 7 d of GH therapy) and how the pattern of GH administration significantly affected the nature of the metabolic responses to immune challenge effected with LPS. Similarly, care must be exercised in comparing data and conclusions between trials and experiments that, from the onset, were intended to address different goals. GH effects on catabolism *per se* must be dealt with and evaluated in a character different from those scenarios concerned with trauma, burns, low-level infection, severe infection with multiorgan failure, viral infection, bacterial infection, *etc.* Likewise, inferences drawn on the increase in immune function driven by GH, such as those describing such phenomena as increased oxidative burst in neutrophils and macrophages with increased pathogen-killing capacity (51, 52), may not be relevant to the response described here for the liver.

Whether the use of GH constitutes a risk to clinical outcome of disease processes is difficult to assess and is confounded in issues of severity of disease and inherent variability in how individuals cope with the immune insults. Excessive NO production has been explored as a mechanistic cause for increased morbidity and mortality during severe endotoxemia and sepsis and certainly may impact outcome scenarios through broad connotations regarding NO effects on cardiovascular function, organ perfusion, and mitochondria-related regulation of energy balance and apoptosis.

In general, the benefit to pharmacological manipulation of NOS activity (competitive and noncompetitive substrate analogs of arginine) as a means of effecting a favorable resolution of severe sepsis may reside in the selectivity of the isoform being inhibited and the timing and duration of NOS inhibitor administration. Selective inhibition of iNOS had been correlated with more favorable outcomes than the administration of more generalized blockers that appear to have a higher degree of action directed toward constitutive forms of NOS (33, 53). The application of these pharmacological agents for this purpose is still guided by principles of timing and dose, not the least of which relate to the location, stage, and level of iNOS induction and activity. Clinical application of this arginine analog strategy has been tried by Bakker *et al.* (54), who reported beneficial effects of iv NOS blocker (N^G-methyl-L-arginine HCl) administration for up to 72 h in cases of septic shock. Interestingly, longer infusions of this drug appear to have been associated with increased risk to a healthy outcome (55). A partial explanation for conflicting results when this approach is tried might relate to the propensity for arginine analogs to interact with multiple biochemical paths in a multitude of tissues other than the intended NOS. In contrast to NOS activity changes that might be achieved through isoform-specific phosphorylations or cofactor associations affecting only that particular enzyme, arginine analog administration differentially impacts several of the isoforms of NOS, albeit with different kinetics (53, 56). In addition, analogs also compete with the amino acid transporter systems (57), and other enzyme pathways, such as the urea cycle (58), significantly impinge on the balance of processes that regulate NO production and its fate.

Finally, a treatment plan for therapeutic intervention in severe disease, sepsis in particular, must minimize potential interactions (drug-drug, P450 metabolism, *etc.*) that might lead to a further destabilization of the patient or subject. The initiation of antibiotic therapy in sepsis has the potential to inadvertently cause a massive disruption of bacterial integrity leading to a sudden burst of endotoxin, and this antibiotic-related burst of LPS has been associated with the class and mechanism of action of the antibiotic (59) as well as the species of microbe present. The LPS-mediated burst has been characterized as detrimental to a positive clinical outcome, even when bacterial counts fall, due to a rapid-onset destabilization of the homeostatic response (60, 61). Certainly this must be acknowledged and dealt with accordingly, with the recognition that the presence of additional factors (*i.e.* GH treatment status) may further exacerbate the host response through the very mechanisms described here for the low-level immune challenge. In cases where lower-level immune challenge stress is present or where this might be anticipated, the subtler manifestations of modulated NO production and protein tyrosine nitration can be exploited from a position where milder (for example, nutritional; see Ref. 25) or selective antioxidant (35) interventions can be effectively combined with traditional therapeutic approaches with fewer interactions with the effects or metabolism of pharmacological compounds.

Acknowledgments

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Address all correspondence and requests for reprints to: Dr. Ted H. Elsasser, United States Department of Agriculture, Agricultural Research Service, Growth Biology Laboratory, B-200, Room 201, Beltsville Agricultural Research Center-East, Beltsville, Maryland 20705. E-mail: elsasser@anri.barc.usda.gov.

Present address for B.N.: Nereus Pharmaceuticals Inc., San Diego, California 92121.

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